Complement and inflammasome overactivation mediates paroxysmal nocturnal hemoglobinuria with autoinflammation

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**Graphical abstract**

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Complement and inflammasome overactivation mediates paroxysmal nocturnal hemoglobinuria with autoinflammation

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Patients with paroxysmal nocturnal hemoglobinuria (PNH) have a clonal population of blood cells deficient in glycosylphosphatidylinositol-anchored (GPI-anchored) proteins, resulting from a mutation in the X-linked gene PIGA. Here we report on a set of patients in whom PNH results instead from biallelic mutation of PIGT on chromosome 20. These PIGT-PNH patients have clinically typical PNH, but they have in addition prominent autoinflammatory features, including recurrent attacks of aseptic meningitis. In all these patients we find a germ-line point mutation in one PIGT allele, whereas the other PIGT allele is removed by somatic deletion of a 20q region comprising maternally imprinted genes implicated in myeloproliferative syndromes. Unlike in PIGA-PNH cells, GPI is synthesized in PIGT-PNH cells and, since its attachment to proteins is blocked, free GPI is expressed on the cell surface. From studies of patients’ leukocytes and of PIGT-KO THP-1 cells we show that, through increased IL-1β secretion, activation of the lectin pathway of complement and generation of C5b-9 complexes, free GPI is the agent of autoinflammation. Eculizumab treatment abrogates not only intravascular hemolysis, but also autoinflammation. Thus, PIGT-PNH differs from PIGA-PNH both in the mechanism of clonal expansion and in clinical manifestations.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell (HSC) disorder characterized by complement-mediated hemolysis, thrombosis, and bone marrow failure (1, 2). Affected cells harbor a somatic mutation in the PIGA gene, essential for the initial step in glycosylphosphatidylinositol (GPI) biosynthesis that occurs in the endoplasmic reticulum (ER) (Figure 1A, top and ref. 3). Loss of GPI biosynthesis results in the defective expression of GPI-anchored proteins (GPI-APs), including complement inhibitors CD59 and DAF/CD55 (Figure 1A, middle). The affected stem cells generate large numbers of abnormal blood cells after clonal expansion that occurs under bone marrow failure. The affected erythrocytes are defective in complement regulation and destroyed by the membrane attack complex (MAC or C5b-9) upon complement activation (1). Eculizumab, an anti-complement component 5 (C5) monoclonal antibody (mAb), has been used to prevent intravascular hemolysis and thrombosis (4, 5). Eculizumab binds to C5 and inhibits its activation and subsequent generation of C5b-9 complexes.

Among more than 20 genes involved in GPI biosynthesis and transfer to proteins, PIGA is X-linked whereas all others are autosomal (6). Because of X-linkage, one somatic mutation in PIGA causes GPI deficiency in both males and females (3). In contrast, 2 mutations are required for an autosomal gene, but the probability of somatic mutations in both alleles at the same locus is extremely low, which explains why GPI deficiency in most patients with PNH is caused by PIGA somatic mutations. Recently, we reported 2 patients with PNH whose GPI-AP deficiency was caused by germ-line and somatic mutations in the PIGT gene localized on chromosome 20q (7, 8). Both patients had a heterozygous germ-line loss-of-function mutation in PIGT, along with loss of the normal allele of PIGT by a deletion of 8 Mb or 18 Mb occurring in HSCs (7, 8). PIGT, forming a GPI transamidase complex with PIGK, PIGS, PIGU, and GPAA1, acts in the transfer of preassembled GPI to
proteins in the ER (Figure 1A, top and ref. 9). In PIGT-defective cells, GPI is synthesized but is not transferred to precursor proteins, resulting in GPI-AP deficiency on the cell surface (Figure 1A, bottom). We showed recently that non–protein-linked, free GPI remaining in the ER of PIGT-defective Chinese hamster ovary (CHO) cells is transported to and displayed on the cell surface (Figure 1A, bottom and ref. 10).

Two reported PNH patients with PIGT defect suffered from recurrent inflammatory symptoms that are unusual in patients with PNH (7, 8). Here, we report 2 more patients with PNH who lost PIGT function via a similar genetic mechanism, and present insights into the expansion of PIGT-defective clones common among 4 patients. We also present the integrated clinical characteristics of these 4 patients and show that PIGT-defective mononuclear leukocytes, but not PIGA-defective mononuclear leukocytes, secreted IL-1β in response to inflammasome activators. Using a PIGT-KO THP-1 cell model, we show that complement activation is enhanced on the surface of PIGT-defective cells leading to MAC-dependent elevated secretion of IL-1β. Against this background, we propose a distinct disease entity, PIGT-PNH.

Results

Case report. Japanese patient J1 (8) and German patients G1 (7), G2, and G3 were diagnosed with PNH at the ages of 68, 49, 65, and 66, respectively. The changes in PNH clone sizes in J1, G1, and G3 after PNH diagnosis are shown in Figure 1B. They were treated with eculizumab, which effectively prevented intravascular hemolysis. Percentage of PNH cells rapidly increased after commencement of eculizumab not only in erythrocytes but also in granulocytes (Figure 1B). We reported that in G1, direct Coombs test–positive erythrocytes appeared after commencement of eculizumab treatment, suggesting extravascular hemolysis (7). Blood cell counts for G1 and G3 are shown in Supplemental Figure 1A. Before the diagnosis of PNH, J1, G1, and G3 had suffered inflammatory symptoms including urticaria, arthralgia, and fever from the ages of 30, 26, and 48, respectively (Table 1). Urticaria in J1 was associated with neutrophil infiltration (8) and that in G3 with a mixed inflammatory infiltrate (Figure 1C). J1 (8) and G3 suffered from recurrent aseptic meningitis characterized by an abundance of neutrophils in cerebrospinal fluid. Following the initiation of eculizumab treatment for hemolysis 3–5 years earlier, J1 and G3 had not suffered any episodes of meningitis (Figure 1D). Urticaria and arthralgia were also ameliorated in all 3 by eculizumab treatment. G2 had severe atherosclerosis, which might be related to autoinflammation (Table 1); however, whether G2 had autoimmune inflammatory symptoms is unclear and could not be confirmed because the patient passed away.

Genetic basis of GPI-AP deficiency. Four patients did not have PIGA somatic mutations but had a germline mutation in one allele of PIGT located on chromosome 20q: patient J1, NM_015937 (8), c.250G>T; patient G1, c.1401+2A>G (7); patient G2, c.761_764del-GAAA; and patient G3, c.197delA (Supplemental Figure 2A; supplemental material available online with this article; https://doi.org/10.1172/JCI123501DS1). These cause E84X, exon 11 skipping, frameshift after G254, and frameshift after Y66, respectively. The functional activities of variant PIGT found in J1 and G1 were reported to be very low (7, 11). Variants in G2 and G3 causing frameshifts should also be severely deleterious to PIGT function. In addition to

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Appendix Figure 1B). We showed recently that non–protein-linked, free GPI on the surface of PIGT-defective cells similar to 20q−MPN and MDS. The deleted region of 8–18 Mb includes the entire myeloid CDR (Figure 2A) and its loss was shown to be causally related to clonal expansion of the affected myeloid cells in these 20q− syndromes (13). In contrast to previous cytogenetic analysis on classical PNH cases that showed no aberrations in 20q (14, 15), one allele of the myeloid CDR was lost in PNH cells of all 4 patients (Supplemental Figure 2B and refs. 7, 8).

The tumor suppressor–like gene L3MBTL1 and the kinase gene SGK2 were detected in both GPI-AP–defective granulocytes from J1 and normal granulocytes (Figure 2C). The transcripts of 2 unimprinted genes, IFT52 and MYBL2, were detected in both GPI-AP–defective granulocytes from J1 and normal granulocytes (Figure 2C, top). The results therefore indicate that the expression of L3MBTL1 and SGK2 is lost in GPI-AP–defective cells in J1 and G1.

The results shown in Figure 2C also indicate that the somatically deleted region in J1 and G1 included active L3MBTL1 and SGK2, so it was in the paternal chromosome. Owing to mRNA expression from patients G2 and G3 not being available, we determined the methylation status of the L3MBTL1 gene using DNA from blood leukocytes, among which the large majority of cells were of the PNH phenotype. L3MBTL1 in G1 and G3 samples was hypermethylated (Figure 2D and Supplemental Figure 3A), indicating that the myeloid CDR allele remaining in their PNH clones was imprinted. In contrast, the G2 sample was hypomethylated. It was reported that, in some MPN patients with myeloid CDR deletion, the remaining allele was hypomethylated; nevertheless, its transcription was suppressed (13). G2 might be in a similar situation; although it was not possible to draw a definitive conclusion on this by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis as the patient had passed away. These results indicate that the loss of expressed myeloid CDR allele is associated with clonal expansion of PIGT-defective cells similar to 20q− MPN and MDS.

Appearance of free GPI on the surface of PIGT-defective cells. PIGA is required for the first step in GPI biosynthesis (17); therefore, no
Figure 1. Clinical features of PIGT-PNH. (A) Schematics of normal and defective biosynthesis of GPI-APs. (Top) In normal cells, GPI is synthesized in the ER from phosphatidylinositol (PI) by sequential reactions and assembled GPI is attached to proteins (orange oval). PIGA acts in the first step whereas PIGT acts in attachment of GPI to proteins. GPI-APs are transported to the plasma membrane (PM). (Middle) No GPI biosynthesis in PNH caused by PIGA defect. (Bottom) Accumulation of free GPI in PNH cells caused by PIGT defect. Non–protein-linked GPI is transported to the PM. (B) Time course of PNH clone sizes in patients G1, G3, and J1. Percentages of PNH cells in monocytes, granulocytes, erythrocytes, and reticulocytes are plotted as a function of time in days. Arrows, start of eculizumab therapy. (C) Examples of urticaria in G3 before the start of the anakinra treatment are shown on the left (chest) and middle (left upper leg); hemoglobinuria in G3 is shown on the right. Brightness was adjusted in the bottom chest image to more clearly show raised skin in the affected area. The pictures were made available by the patient. (D) Clinical courses of G3 in comparison to J1 (Figure 1 in ref. 8 was modified with additional data) including effective treatments. G3 (top) had meningitis 19 times between 62–65 years of age. Eculizumab therapy started at 66 years of age after a severe hemolysis. J1 (bottom) had meningitis 121 times between 53–69 years of age when eculizumab therapy started. Downward green arrows, onset of urticaria and/or arthralgia; blue middle height bars, meningitis; orange short bars, hemolysis; orange long bars, severe hemolysis; horizontal arrows of various lengths, treatment periods of effective therapies (anakinra and canakinumab were given with prednisolone); upward arrows with number and asterisk, serum samples taken for cytokine and other protein determination.
GPI intermediate is generated in PIGA-defective cells (Figure 1A, middle). PIGT is involved in the attachment of GPI to proteins. GPI is synthesized in the ER, but is not used as a protein anchor in PIGT-defective cells (Figure 1A, bottom). We used T5 mAb that recognizes free GPI, but not protein-bound GPI, as a probe to characterize free GPI (Figure 3A and refs. 18, 19; see Methods for epitope and other characteristics of this antibody). Using T5 mAb in Western blotting and flow cytometry, we first compared PIGT-defective CHO cells with PIGL-defective CHO cells, in which an early GPI biosynthetic step is defective, like in PIGA-defective cells. T5 mAb revealed a strong band of free GPI at a position corresponding to approximately 10 kDa in lysates of PIGT-defective cells but not of PIGL-defective cells (Figure 3B). DAF and CD59 were not detected in either mutant cell, confirming that non-GPI-anchored precursor proteins were degraded (Figure 3B and ref. 20). T5 mAb stained the surface of PIGT-defective CHO cells but not PIGL-defective cells, confirming that free GPI transported to the cell surface is detectable by T5 mAb (Figure 3C).

We then analyzed blood cells from J1, G1, and G3, and from patients with PIGA-PNH by flow cytometry. All 4 patients had PNH-type blood cells defective in various GPI-APs (Figure 3, D–F, and Supplemental Figure 4, A and B). Erythrocytes from J1, G1, and G3 contained 3%, 84%, and 60% PNH cells, respectively, and a sizable fraction of them (36%, 87%, and 87%, respectively) were stained by T5 mAb (Figure 3D). J1 had PNH cells in granulocytes (81%), monocytes (87%), and B lymphocytes (54%), but not T lymphocytes (<2%), as revealed by anti-CD59 and GPI-binding probe fluorescence-labeled nonlytic aerolysin (FLAER) (21). Affected monocytes and B lymphocytes were strongly stained by T5 mAb, whereas affected granulocytes were weakly but clearly stained (Figure 3E).
stain PIGT-PNH cells. Aerolysin specifically binds to the GPI moiety of some but not all GPI-APs, and requires simultaneous association with N-glycan for high-affinity binding (25–27). Our results indicate that FLAER binds to protein-bound GPI but not to free GPI.

We next investigated whether GPI-AP–defective clone was present in the stage only with autoinflammation. After determining the break points causing the deletion of 18 Mb in J1 (Supplemental Figure 5), we quantitatively analyzed blood DNA samples for the presence of the break. It was estimated that a relative level of the break in the sample taken in a stage with autoinflammation only (#1) was approximately 10% of a level in the sample taken 1 month after start of eculizumab therapy (#2), which contained 29% PNH cells (Figure 3G). Therefore, approximately 3% of total leukocytes obtained 4 months before the onset of recurrent hemolysis were GPI-AP–defective cells.

Figure 2. Genetic abnormalities in patients with PIGT-PNH. (A) PIGT mutations in GPI-AP–positive (GPI +) and –defective (GPI –) cells from patients with PIGT-PNH. (Top) GPI + cells from patients J1, G1, G2, and G3 had a germline PIGT mutation (triangle) in the maternal (M) allele. Two maternally imprinted genes, L3MBTL1 and SGK2, within myeloid common deleted region (CDR) are expressed from the paternal (P) allele. Solid and broken red double arrows, P and M alleles of myeloid CDR, respectively. (Bottom) GPI blood cells from PIGT-PNH patients had an 8 Mb to 18 Mb deletion spanning myeloid CDR and PIGT and/or PIGU in the P chromosome 20q leading to losses of expression of L3MBTL1 and SGK2 genes (dotted boxes). (B) A 1.9-Mb region in chromosome 20q spanning PTPRT gene to OSER1 gene is termed myeloid common deleted region. PIGT and PIGU genes are approximately 1.2 Mb telomeric and 7.4 Mb centromeric to the myeloid CDR, respectively. L3MBTL1 and SGK2 genes marked # are maternally imprinted. (C) qRT-PCR analysis of genes within myeloid CDR in GPI-AP–defective granulocytes from J1 and granulocytes from a healthy control (top) and whole blood cells from G1 and a healthy control (bottom). L3MBTL1 and SGK2, maternally imprinted genes; IFT52 and MYBL2, nonimprinted genes. Relative expression is determined taking means of ABL levels as 1 (J1) or of GAPDH as 1 (G1). Blue bars, cells from J1 and G1; orange bars, cells from healthy individuals; * indicates below detection limits. Mean of duplicate (J1) and triplicate (G1) samples in 1 of the 2 independent experiments. Mean RQ max values for J1 samples were 0.15 (IFT52) and 0.17 (MYBL2), and for normal control samples were 0.034 (L3MBTL1), 0.003 (SGK2), 0.078 (IFT52), and 0.002 (MYBL2). Mean RQ max values for G1 samples were 0.01 (SGK2), 0.004 (L3MBTL1), and 0.01 (GAPDH), and for normal control samples were 0.08 (SGK2), 0.22 (L3MBTL1), and 0.002 (GAPDH). (D) Methylation status of the CpG islands in L3MBTL1 in G1, G2, and G3. Red, methylated CpG islands; pink, unmethylated CpG islands; gray, unknown CpG islands. Bisulfite sequencing data are shown in Supplemental Figure 3A.

and Supplemental Figure 4B). Normal populations in granulocytes, monocytes, and B lymphocytes were not stained by T5 mAb (Figure 3E). Similar results, showing strong T5 staining of affected monocytes and granulocytes, were obtained with leukocytes from G1 and G3 (Figure 3F). In contrast, PNH cells from PIGA-PNH patients and cells from healthy individuals were not stained by T5 mAb (Figure 3D). Free GPI might be transferred from PNH cells to WT erythrocytes in vivo (22, 23), although the exact mechanism involved needs to be clarified. Thus, the surface expression of the T5 mAb epitope is specific for PIGT-defective cells and T5 mAb is useful to diagnose PIGT-PNH. It was noted that FLAER, which is conveniently used to stain cell-surface GPI-APs and to determine the affected cells in patients with PNH (21, 24), did not stain PIGT-PNH cells. Aerolysin specifically binds to the GPI moiety of some but not all GPI-APs, and requires simultaneous association with N-glycan for high-affinity binding (25–27). Our results indicate that FLAER binds to protein-bound GPI but not to free GPI.

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Inflammasome- and complement-mediated autoinflammation, a feature of PIGT-PNH. IL-18 levels were elevated in serum samples taken from J1 before and after the commencement of eculizumab therapy (Table 2), suggesting that this phenomenon was not dependent upon C5 activation. Serum amyloid A was also elevated before eculizumab therapy, but was within the normal range after the commencement of eculizumab therapy (Table 2), suggesting that the elevation was dependent upon C5 activation. In G3, increased levels of soluble IL-2 receptor and thymidine kinase before, but not after, the start of eculizumab therapy suggested autoinflammation only (4 months before the onset of recurrent hemolysis); #2. DNA from granulocytes (29% of cells were GPI-AP–defective) taken 1 month after start of eculizumab therapy. (Right) Relative levels of the break in samples #1 and #2 by setting the level in #2 as 1. Data are shown in mean of triplicate samples in 1 experiment. Mean RQ max values for #1 and #2 samples were 0.092 and 1.11, respectively.

To investigate the roles of complement in inflammasome activation in PIGT-PNH, we switched to a model cell system because patients’ blood cells were easily damaged in vitro under conditions of complement activation. PIGT-KO and PIGA-KO cells were generated from human monocyte THP-1 cells (Supplemental Figure 6A) and were differentiated to macrophages. They showed IL-1β response comparable to those of authentic inflammasome activators (Supplemental Figure 6B). To analyze the inflammasome response to activated complement, these THP-1–derived macrophages were stimulated with acidified serum (AS), which causes activation of the alternative complement pathway. PIGT-KO and PIGA-KO cells but not WT cells secreted IL-1β (1221.8 ± 91.6 pg/mL, 568.2 ± 101 pg/mL, and 23.7 ± 2.2 pg/mL for PIGT-KO, PIGA-KO, and WT cells, respectively) (Figure 5A). This result is consistent with impaired complement regulatory activities on PIGT-KO and PIGA-KO cells, and normal complement regulatory activity on WT cells. PIGT-KO cells secreted approximately twice as much IL-1β as PIGA-KO cells (P < 0.01). However, IL-1β production returned to near the WT cell level after the transfection of PIGT and PIGA cDNAs into PIGT-KO and PIGA-KO cells, respectively (Figure 5B). The levels of IL-1β mRNA and protein were comparable in WT, PIGT-KO, and PIGA-KO cells (Supplemental Figure 7, A and B). Therefore, knock out of PIGN enhanced the secretion but not the generation of IL-1β.

Heat inactivation of complement and the addition of anti-C5 mAb to AS almost completely inhibited IL-1β secretion (Figure 5A). These results indicate that IL-1β secretion requires the activation of C5 on PIGT-KO and PIGA-KO cells. The activation of C5 leads to 2 biologically active products, C5a and MAC (29). To address which of these is important for IL-1β secretion, cells were treated with the C5aR antagonist W-54011 (30) or anti-C5aR mAb to AS almost completely inhibited IL-1β secretion (Figure 5A). These results suggest that autoinflammation is a feature of PIGT-PNH.

We next compared mononuclear cells from J1, patients with PIGA-PNH, and healthy donors for IL-1β production upon stimulation by NLRP3-inflammasome activators (28). Cells from 3 PIGA-PNH patients secreted only very low levels of IL-1β after stimulation by Pam3CSK4 (TLR2 ligand) and ATP or monosodium urate (MSU) (Figure 4A, right, and Figure 4B). In contrast, cells from J1 secreted 45–60 times as much IL-1β and the levels were even higher than those from healthy control cells (Figure 4A, left, and Figure 4B). A similar difference between PIGT- and PIGA-defective cells was seen upon stimulation by lipoteichoic acid (LTA); another TLR2 ligand) and ATP or MSU (Figure 4B). Low IL-1β response of PIGA-defective cells was predicted because they lack CD14, a GPI-anchored coreceptor of TLRs. However, PIGT-defective cells also lacking CD14 showed a strong IL-1β response. These results indicate that NLRP3 inflammasomes are easily activated and support the idea that the presence of non–protein-linked free GPI is associated with efficient activation of NLRP3 inflammasomes, contributing to autoinflammatory symptoms in PIGN-PNH.

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Table 2. J1: cytokines and other proteins in serum samples

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>*1, interval</th>
<th>*2, meningitis</th>
<th>*3, hemolysis</th>
<th>*4, 2.5 years after start of eculizumab</th>
<th>*5, 3.5 years after start of eculizumab</th>
<th>*6, 4.5 years after start of eculizumab</th>
<th>Normal range</th>
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<tr>
<td>IL-18 (pg/mL)</td>
<td>1410</td>
<td>797</td>
<td>972</td>
<td>1150</td>
<td>&lt;211.0</td>
<td>&lt;211.0</td>
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<tr>
<td>IL-1 (pg/mL)</td>
<td>0.291</td>
<td>&lt;0.125</td>
<td>4.670</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.928</td>
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<tr>
<td>IL-1RA (pg/mL)</td>
<td>716</td>
<td>323</td>
<td>27500</td>
<td>239</td>
<td>85.6–660</td>
<td>85.6–660</td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>127.0</td>
<td>4160</td>
<td>2.30</td>
<td>2.94</td>
<td>6.32</td>
<td>6.32</td>
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<tr>
<td>Serum amyloid A (μg/mL)</td>
<td>175.8</td>
<td>2433.0</td>
<td>282.6</td>
<td>&lt;2.5</td>
<td>8.0</td>
<td>&lt;8.0</td>
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<tr>
<td>LDH (IU/L)</td>
<td>291</td>
<td>249</td>
<td>3004</td>
<td>157</td>
<td>165</td>
<td>169</td>
<td>120–250</td>
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</table>

IL-1RA, IL-1 receptor antagonist. *Serum samples correspond to asterisked numerals in Figure 1D, representing samples from patient J1 during the indicated health events.

Table 3. G3: cytokines and other proteins in serum samples

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>*1, meningitis</th>
<th>*2, meningitis</th>
<th>*3, meningitis</th>
<th>*4, meningitis</th>
<th>*5, six months after start of eculizumab</th>
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<td>Thymidine kinase (U/L)</td>
<td>101</td>
<td>33.6</td>
<td>43.8</td>
<td>Not done</td>
<td>Normal</td>
<td>&lt;5</td>
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<tr>
<td>Soluble IL-2 receptor (U/mL)</td>
<td>1750</td>
<td>1345</td>
<td>3026</td>
<td>1237</td>
<td>Normal</td>
<td>158–635</td>
</tr>
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</table>

*Serum samples correspond to asterisked numerals in Figure 1D, representing samples from patient G3 during the indicated health events.

Role in the secretion of IL-1β. It is also suggested that free GPI plays some role in complement activation, leading to the enhanced binding of C3b fragments and MAC formation.

To determine whether the structure of free GPI (presence or absence of Gal capping) affects complement activation and subsequent IL-1β secretion, we knocked out SLC35A2 in PIGT-KO THP-1 cells. PIGT-SLC35A2 double-KO THP-1 cells were strongly stained by T5 mAb as expected (Supplemental Figure 6C). The binding of both C3b fragments and MAC increased approximately 5 times after SLC35A2 KO (Figure 5F). Concomitantly, the secretion of IL-1β more than doubled (Figure 5G). These results indicate that the structure of free GPI influenced complement activation efficiency and subsequent IL-1β secretion.

Finally, we asked whether only the alternative pathway is involved in binding of C3b fragments on PIGT-KO and PIGA-KO THP-1 cells, or the lectin and/or the classical pathway is also involved. For this, cells were stained with anti-

C4d mAb after treatment with acidified serum because activation of either pathway would result in binding of C4b, which is in turn converted to C4d (31, 32). C4d fragments were bound on PIGT-KO cells and the binding was inhibited by 100 mM mannose by approximately 50% but not by N-acetylgalcosamini (Figure 5H, top, and Supplemental Figure 8D). Binding of C3b fragments on PIGT-KO cells was also inhibited by mannose by approximately 60% but not by N-acetylgalcosaminase (Figure 5H, bottom). These results suggest that the lectin pathway was activated on PIGT-KO THP-1 cells, accounting for at least 60% of C3b fragments. C4d fragments were bound on PIGA-KO cells at lower levels (60%–70% of PIGT-KO cell levels), which were not inhibited by either N-acetylgalcosaminase or mannose. Mannose, but not N-acetylgalcosamine, mildly inhibited binding of C3b fragments on PIGA-KO cells (Figure 5H). It is unclear at the moment whether the classical pathway was involved in residual C4d binding on PIGT-KO cells in the presence of 100 mM mannose or in C4d binding on PIGA-KO cells.

Discussion

We studied patients with PNH caused by PIGT mutations. Because PNH caused by PIGT mutations is characterized by auto-inflammatory symptoms, we propose that they represent a new disease entity, PIGT-PNH. Patients with PIGT-PNH had a germline heterozygous mutation in the PIGT gene in combination with somatic deletion of the normal PIGT gene. Germline PIGT mutations were reported in patients with inherited GPI deficiency (IGD) (Supplemental Table 2), which is characterized by developmental delay, seizures, hypotonia, and typical facial dysmorphism (11, 33–39). Inflammatory symptoms and intravascular hemolysis were not reported in IGD patients with PIGT mutations. They had either partial loss-of-function homozygous mutations, or combinations of a partial loss-of-function mutation and a null or nearly null mutation. Therefore, cells from the patients with IGD have only partially reduced PIGT activities and express only partially reduced levels of CD59 and DAF/CD55, and may have free GPI only to a small extent. In contrast, both germline and somatic mutants in PIGT-PNH were functionally null or nearly null (Table 1). Therefore, the affected cells from PIGT-PNH patients lost CD59 and DAF/CD55 severely or completely and had high levels of free GPI. Interestingly, the same mutation c.250G>T (p.E84X) was found in J1 and 2 Japanese patients with IGD (11, 37) who were not related to each other. PIGT-PNH patient J1 and the mothers of 2 IGD patients from families 2 and 7 had the same heterozygous mutation (Table 1 and Supplemental Table 1). Therefore, the affected cells from PIGT-PNH patients lost CD59 and DAF/CD55 severely or completely and had high levels of free GPI. Interestingly, the same mutation c.250G>T (p.E84X) was found in J1 and 2 Japanese patients with IGD (11, 37) who were not related to each other. PIGT-PNH patient J1 and the mothers of 2 IGD patients from families 2 and 7 had the same heterozygous nonsense PIGT mutation (Table 1 and Supplemental Table 2). These mothers were healthy and no inflammatory symptoms were reported for them (11, 37), suggesting that autoinflammation of J1 was not caused by haploinsufficiency of PIGT but was initiat-
Japanese may have this variant (40). It is, therefore, highly likely that J1 inherited the PIGT variant.

The expansion of PIGA-defective clones in PNH is caused by selective survival under autoimmune bone marrow failure in most cases (41–43). This mechanism was first formulated by Rotoli and Luzzatto in 1989 (41). In few cases, clonal PNH cells acquired benign tumor characteristics by additional somatic mutations (44–46). In contrast, none of the patients with PIGT-PNH had documented bone marrow failure (Supplemental Figure 1A and refs. 7, 8). In addition, the myeloid CDR is lost in PIGT-defective clones in PIGT-PNH, similar to the case in clonal cells in myeloproliferative 20q− syndromes. The causal relationship between the myeloid CDR loss in PIGT-PNH and clonal expansion needs to be proven, particularly because boosted lineages under L3MBTL1 and SGK2 loss differ ed after the somatic loss of the other PIGT copy occurred. Inheritance of the germline PIGT mutations in PIGT-PNH patients is not formally proven because DNA samples were not available from their families. A reported allele frequency of the germline variant PIGT of J1 and 2 Japanese families of IGD, c.250G>T (p.E84X), is 0.0002316 in the East Asian population, suggesting that 55,000

### Table 4. PIGA-PNH: cytokines and other proteins in serum samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
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<td>IL-18 (pg/mL)</td>
<td>288</td>
<td>289</td>
<td>263</td>
<td>443</td>
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<td>Serum amyloid A (μg/mL)</td>
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<td>7.1</td>
<td>6.8</td>
<td>8.3</td>
<td>&lt;8.0</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>633</td>
<td>1190</td>
<td>923</td>
<td>1216</td>
<td>103–229</td>
</tr>
</tbody>
</table>

Data for 4 patients without eculizumab therapy.
between in vitro study (13) and PIGT-PNH patients, and because platelet and leukocyte counts were not much changed in PIGT-PNH patients (Supplemental Figure 1A). Complement dysregulation, which is present in PIGT-PNH but not in myeloproliferative 20q− syndromes, might modulate blood cell profiles. Nevertheless, this unique deletion occurs in PIGT-PNH but not in PIGA-PNH. Taking these findings together, it is likely that the mechanism of clonal expansion for PIGT-PNH is distinct from that for PIGA-PNH cells (see models in Supplemental Figure 3B).

Whereas PIGT-PNH shares intravascular hemolysis and thrombosis with PNH, PIGT-PNH is characterized by autoinflammatory symptoms including recurrent urticaria, arthralgia, and aseptic meningitis. PIGT-PNH first manifested with autoinflammatory symptoms alone and symptoms of PNH became apparent many years later (Table 1). It is possible that different clinical symptoms appear depending on the size of the PIGT-defective clone. When the clone size is small, autoinflammation but not PNH may occur and, when the clone size becomes sufficiently large, PNH may become apparent. The idea that the CLT-defective clone is smaller when only autoinflammation is seen was supported by analysis of IL-1β secretion from THP-1 cells after a 5-hour incubation and analyzed for IL-1β by ELISA. Mean ± SD of 3 independent experiments. (B) Reductions of IL-1β secretion by transfection of PIGT and PIGA DNAs into PIGT-KO and PIGA-KO cells (PIGT-KO+T and PIGA-KO+A, respectively). Cells differentiated by PMA were either left untreated (PMA) or incubated with AS (AS) under similar conditions as described in A, and supernatants analyzed for IL-1β. Mean ± SD of 3 independent experiments. (C) Effect of inhibiting C5arα on IL-1β secretion from THP-1-derived macrophages. Cells were incubated with AS alone (no treat), or AS containing C5arα antagonist (W-54011) or anti-C5αR mAb. Supernatant was collected after 5 hours and analyzed for IL-1β by ELISA. Mean ± SD of duplicate samples from 2 independent experiments. (D) Detection of C3b fragments (left) and MAC (right) by flow cytometry on PMA-differentiated THP-1 macrophages after incubation with AS. Geometric mean fluorescence intensity of medium-treated cells was subtracted from that of AS-treated cells. Mean ± SD of 3 independent experiments. (E) IL-1β secretion from PIGT-KO THP-1 macrophages stimulated with C6- or C7-depleted AS. PIGT-KO THP-1 macrophages were incubated with AS, C6-depleted AS (-/C6de), C6de restored by C6 (+C6/C6de), C7-depleted AS (-/C7de), or C7de restored by C7 (+C7/C7de). Supernatant was collected after overnight incubation. Mean ± SD of triplicate samples from 2 independent experiments (normal and C6-depleted sera) and 1 experiment (C7-depleted serum). (F) Binding of C3b fragments (left) and MAC (right) on PIGT-KO and PIGT-SLC35A2 double KO THP-1 macrophages after AS treatments. (G) IL-1β secretion from PIGT-KO and PIGT-SLC35A2 double KO THP-1 macrophages after AS treatments. (H) Binding of C4d(top) and C3b fragments (bottom) on PIGT-KO and PIGA-KO THP-1 macrophages after AS treatments and inhibition by mannose. Two-tailed Student’s t test was used for analysis. Mean ± SD of 3 independent experiments.

to easy activation of NLRP3 inflammasomes in monocytes and autoinflammatory symptoms (47), further suggesting that inflammasomes are activated in monocytes from PIGT-PNH patients. It was reported that autoinflammation occurs in patients having mosaicism with NLRP3-mutant cells even when the mutant clone size is small (frequency of mutant allele in whole-blood cells being 4.3% to 6.5%) (48). This is relevant to the symptoms/clone size relationship in PIGT-PNH as discussed above.

C5 activation must be involved in the autoinflammatory symptoms in PIGT-PNH because they were suppressed by eculizumab. It is important to consider GPI-AP deficiency for patients with recurrent autoinflammatory symptoms such as aseptic meningitis even when PNH symptoms are absent because eculizumab may be effective for such cases. Because DAF and CD59 are missing on PIGT-defective monocytes, C5a and MAC might be generated once complement activation has been initiated. It was reported that subarachnoidal application of C5a in rabbits and rats induced acute experimental meningitis (49). Various types of myeloid cells are present in the central nervous system (reviewed in ref. 50). If C5 activation occurs on some of those cells lacking complement regulatory function and C5a is generated, aseptic meningitis might ensue.

The involvement of complement in inflammasome activation has been shown in various blood cell systems (51–54). Indeed, in the THP-1 cell model system, IL-1β secretion was induced by complement in both PIGT-KO and PIGA-KO cells and more strongly in PIGT-KO cells, mainly through MAC formation (Figure 5, A, B, and E). PIGT-PNH mononuclear cells were activated by conventional stimulators of inflammasomes similar to or even stronger than healthy control cells. Because blood mononuclear cells were easily lysed by acidic serum, the effect of complement on inflammasome activation in mononuclear cells could not be addressed. Taking the obtained findings together with the results for THP-1 cells, we speculate that PIGT-deficient monocytes show an enhanced inflammasome response when complement is activated. How free GPI is involved in inflammasome and complement activation needs to be further clarified to fully understand the mechanistic basis of PIGT-PNH.

PIGU is an essential component of GPI transamidase, forming a protein complex with PIGT, PIGS, PIGK, and GAPA1 (Figure 1A and ref. 55). PIGU-defective cells do not express GPI-APs on their surface (55). The PIGU gene is localized at approximately 7.4 Mb centromeric to the myeloid CDR (Figure 2, A and B), and regions of somatic deletions of 18 Mb and 12 Mb in GPI-AP–defective cells from J1 and G2, respectively, included the entire PIGU gene as well as myeloid CDR and PIGT gene. The levels of PIGU protein in these cells would be around half of the normal levels. It appears unlikely that the 50% reduction in PIGU has a significant impact on these cells. The levels of PIGT protein in the same cells would be zero or very low because mutations in the remaining PIGT gene are a nonsense mutation (E84X) in J1 and a frameshift mutation (frameshift after G254) in G2. For any remaining PIGT protein, half of the normal amounts of PIGU protein would be excessive for making the GPI transamidase complex. However, it is conceivable that, if a similar somatic deletion including PIGU and myeloid CDR occurs in a hematopoietic stem cell of an individual who bears a germline PIGU loss-of-function mutation, PNH with autoinflammation caused by PIGU mutation might occur.
Eculizumab was used for patients with PIGT-PNH. There are similarities and differences between PIGT-PNH and PNH against treatments with eculizumab. Similar to PNH, eculizumab was effective in preventing intravascular hemolysis in PIGT-PNH. Also similar to PNH, eculizumab caused appearance of direct Coombs-positive erythrocytes in patient G1, suggesting an induction of extravascular hemolysis (7). Patient G2 had residual hemolysis after start of eculizumab therapy, also suggesting extravascular hemolysis (Table 1). PIGT-KO THP-1 cells accumulated higher levels of C3b fragments upon complement activation (Figure 5D) and the affected erythrocytes from PIGT-PNH patients express free GPI (Figure 3D). Whether the free GPI-bearing PNH erythrocytes from PIGT-PNH patients accumulate more C3b fragments than PNH erythrocytes from PIGA-PNH patients and hence are more prone to extravascular hemolysis is yet to be clarified.

A difference is that percentage of PIGT-PNH cells increased not only in erythrocytes but also in granulocytes after treatment with eculizumab (Figure 1B). It is well known that percentage of PNH erythrocytes increases after eculizumab treatment because intravascular destruction of PNH erythrocytes is prevented by eculizumab (4). However, a sharp increase in percentage of PNH granulocytes seen in G1, G3, and J1 has not been reported in PIGA-PNH (4). A possible explanation for the difference is that PIGT-PNH granulocytes might have been destroyed in the blood by complement-dependent mechanisms. MAC formation was much higher on PIGT-KO THP-1 cells than on PIGA-KO cells (Figure 5D). If levels of MAC formation on PIGT-defective granulocytes are similarly high, MAC-mediated cell lysis might occur and eculizumab might prevent it, leading to an increase in the percentage of PNH granulocytes. Another possibility is that the commencement of eculizumab treatment was only coincident with the active phase of clonal expansion. Because hemolysis becomes apparent only when clone size has increased, it is still possible that use of eculizumab is not causally related to increase of PNH granulocytes but that eculizumab treatment was done during active clonal expansion.

Eculizumab was effective in preventing recurrent autoimmune symptoms of PIGT-PNH. After start of eculizumab therapy, aseptic meningitis has not occurred in patients G3 and J1, and other symptoms such as urticaria and arthralgia were also prevented (Figure 1D and ref. 8). These autoimmune symptoms, therefore, are dependent upon C5 activation. Results with PIGT-KO THP-1 cells indicated that IL-1β secretion after complement activation was mediated by MAC rather than C5a (Figure 5, C and E). Patient J1 had elevated levels of IL-18 and serum amyloid A (Table 2). After start of eculizumab, serum amyloid A level turned normal whereas IL-18 levels remained 4–5 times of the upper limit of normal. IL-18 secretion in PIGT-PNH, therefore, is not dependent upon terminal complement activation. C3 activation on the surface of PIGT-defective cells was higher than that on PIGA-defective cells, as suggested by experimental results with PIGT-KO and PIGA-KO THP-1 cells (Figure 5, D and E). It appeared that the activation of the lectin pathway was enhanced, leading to elevated levels of C3b fragment binding and MAC formation (Figure 5, D and H). The C4d binding on PIGT-KO cells was significantly inhibited by mannose, suggesting that recognition of mannose residues in free GPIs by complement lectins might be involved in the lectin pathway activation. It seems likely that enhanced C3 activation is involved in the elevation of serum IL-18 levels because C3 activation continues on PIGT-defective cell-surface during eculizumab therapy. Causal relationship between free GPIs and C3 activation, and the molecular mechanisms involved, needs to be clarified to know whether earlier steps in complement activation are involved in pathogenesis of PIGT-PNH.

**Methods**

**Blood samples and flow cytometry.** Peripheral blood samples were obtained from patients J1 (8), G1 (7), G2, and G3 with PIGT-PNH, and 6 patients with PNH after written informed consent. Peripheral blood leukocytes (Supplemental Figure 1B), erythrocytes, and reticulocytes were stained for GPI-APs by FLAER (Cederlane), anti-CD14 (clone MOP9, BD Biosciences), anti-CD16 (clone 3G8, BioLegend), anti-CD24 (clone ML5, BioLegend), anti-CD48 (BJ40, BioLegend), anti-CD59 (clone 5H8) (56), or anti-CD66b (clone 80H3, Beckman Coulter Immunotech). T5-4E10 mAb (T5 mAb) against free GPI of Toxoplasma gondii was a gift from J. F. Dubremetz (18). T5 mAb is now available from BEI Resources, NIAID, NIH (Bethesda, MD). T5 mAb recognizes mammalian free GPI bearing N-acetylgalactosamine (GalNAc) side-chain linked to the first mannose (Figure 3A and ref. 19). T5 mAb does not bind to free GPI when galactose (Gal) is attached to GalNAc. Therefore, reactivity of T5 mAb to free GPI is affected by an expression level of Gal transferase that attaches Gal to the GalNAc. Other antibodies used were PE-anti-CD55/DAF (clone IA10, BD Biosciences), PE-anti-CD88/C5aR (BioLegend), and anti-TfR (clone H68.4, Thermo Fisher Scientific). Cells were analyzed by a flow cytometer (MACSQuant Analyzer VYB or FACSCalibur) and FlowJo software.

**DNA and RNA analyses.** Granulocytes with PNH phenotype were separated from normal granulocytes by cell sorting after staining by FLAER. DNA was analyzed for mutations in genes involved in GPI-AP biosynthesis by target exome sequencing, followed by confirmation by Sanger sequencing (7). DNA was also analyzed by array comparative genomic hybridization for deletion (7). Methylation status of CpG was determined by bisulfite sequencing and a SnuPE assay (13). Total RNA was extracted with the RNasy Mini Kit (Qiagen) including DNase digestion and DNA cleanup, and reverse transcription was performed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Levels of LMBTL1, SGK2, IFT52, MYBL2, ABL, and GAPDH mRNAs were analyzed by quantitative real-time PCR (Supplemental Table 1).

**Cell lines.** PIGT-defective CHO cells and PIGL-defective CHO cells were reported previously (11, 57). CRISPR/Cas9 system was used to generate PIGT-KO and PIGA-KO human monocytic THP-1 cells (ATCC) (Supplemental Table 1 for guide RNA sequences). Knockout cells were FACS sorted for GPI-AP-negative cells. Each knockout cell was rescued by transfection of a corresponding cDNA. SGC552 gene was knocked out in PIGT-KO THP-1 cells by CRISPR/Cas9 system.

**Inflammassome activation and IL-1β measurements.** Toll-like receptor 2 (TLR2) ligands Pam, CSK4 and Staphylococcus aureus LTA are from InvivoGen (28, 58). ATP and MSU for activating inflammasomes are from Enzo Life Sciences and InvivoGen (28, 59). Peripheral blood mononuclear cells were stimulated by Pam, CSK4 or LTA for 4 hours at 37°C and after washing by ATP or MSU for 4 hours at 37°C. IL-1β ELISA kit (BioLegend) was used to measure IL-1β secreted into the supernatants. Polyclonal rabbit anti-IL-1β antibody for Western blotting was from Cell Signaling Technology. PIGA-KO, PIGT-KO, and WT THP-1 cells were differentiated into adherent macrophages in complete RPMI.
1640 medium containing 100 ng/mL phorbol 12-myristate 13-acetate (PMA; InvivoGen) for 3 hours, and then with fresh complete medium overnight (60). For stimulation, medium was replaced with serum-free medium with Pam,C5K (200 ng/mL), followed 4 hours later by ATP stimulation for 4 hours (5 mM).

Stimulation of THP-1-derived macrophages with complement. As a source of complement, whole blood was collected from healthy donors after written informed consent, and serum separated, aliquoted, and stored at -80°C prior to use. Inactivation of complement was carried out by heating serum at 56°C for 30 minutes. To prepare AS that allows activation of the alternative pathway on the cell surface, 21 volumes of serum was mixed with 1 volume of 0.4M HCl to have a pH of approximately 6.7. C6- and C7-depleted sera and purified C6 and C7 proteins were purchased from Complement Technology. Differentiated cells were stimulated with acidified normal serum, or acidified C6-depleted and C7-depleted sera, and those reconstituted with C6 and C7, respectively, at 37°C for 5 hours, and secreted IL-1b was measured by ELISA. C5 was inhibited by addition of 35 μg/mL anti-C5 mAb (eculizumab, Alexion Pharmaceuticals).

For ex vivo blockade of human C5aR, anti-human C5aR or nonpeptide C5aR antagonist W-54011 (5 μM, Merck Millipore) (30) was used. Complement C3 and C4 fragments and MAC deposited on the cells were measured by flow cytometry. THP-1 cells were suspended in 20 μL FACS buffer (PBS, 1% BSA, 0.05% sodium azide) with 1:20 human TruStain FcX (Fc receptor blocking solution) at room temperature for 10 minutes. Cells were stained with anti-C3/C3b/iC3b/C3d mAb (clone 1H8, BioLegend), anti-C4d mAb (clone 1D11, Hycult Biotech) or rabbit anti-human SC5b-9 (MAC) polyclonal antibodies (Complement Technology) in FACS buffer. After washing twice, cells were incubated with the PE-conjugated goat anti-mouse IgG (BioLegend) or Alexa Fluor488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) secondary antibody. The anti-human SC5b-9 polyclonal antibodies positively stained PMA-differentiated THP-1 cells without incubation in AS. The same antibodies did not stain similarly differentiated PIGT-KO and PIGA-KO THP-1 cells, suggesting that the antibody product contained antibodies reacted with some GPI-AP expressed on THP-1–derived macrophages (Supplemental Figure 8, B and C). Because of this reactivity to non-MAC antigen(s), the anti-SC5b-9 antibodies were used for PIGT-KO and PIGA-KO cells but not for WT cells in experiments shown in Figure 5, D and F. To inhibit the lectin pathway, serum was mixed with mannose or N-acetylgallosaminogluconic acid (final concentration of 100 mM) before acidification (61).

Statistical analyses. All experiments with THP-1 cells were performed at least 3 times. All values were expressed as the mean ± SD of individual samples. For 2-group comparisons between PIGT-KO and PIGA-KO cells, 2-tailed Student’s t test was used. P values less than 0.05 were considered statistically significant.

Study approval. This study was approved by the institutional review boards of Osaka University (approval number 681), University of Ulm (approval numbers 279/09 and 188/16), and University of Berlin (approval number EA2/077/12).

Data sharing statement. All data supporting the findings are available from the corresponding authors.

Author contributions

BH, YM, NI, HS, PMK, and TK designed research. YM, MO, AK, TH, S Murata, TE, MJ, RF, and AH performed research. BH, MK, MA, S Murase, YU, and NK acquired the data. BH, YM, MO, MK, JN, YK, NK, HS, and PMK analyzed data. BH, YM, MO, HS, PMK, and TK wrote the paper. Three authors sharing the first author position are in alphabetical order.

Acknowledgments

We thank Morihisa Fujita (Jiangnan University), Tatsutoshi Nakahata (Kyoto University), Hidenori Ohnishi (Gifu University), Tatsuya Saitho (Tokushima University), and Yusuke Maeda (Osaka University) for discussion; Jean-Francois Dubremetz (Montpellier University) for T5-4E10 mAb; Lucio Luzzatto (Muhimbili University) for critical reading and editing advice on this manuscript; Keiko Kinoshita (Osaka University), Kana Miyanagi (Osaka University), Saori Umeshita (Osaka University), and Miguel Rodriguez de los Santos (University of Ulm) for technical help; Lisa A. Gerdes (Munich University) for collaboration regarding patient G3; and the patients for providing blood samples and pictures. We thank Edanz (www.edanzediting.co.jp) for editing the English text of a draft of this manuscript. This work was supported by the Japan Society for Promotion of Sciences and The Ministry of Education, Culture, Sports, Science and Technology of Japan KAKENHI grants (JP16H04753 and JP17H06422) to TK, and a grant from the Japan Society of Complement Research to YM.

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